Amendment filed on 02/23/2004

RL; 3/22/2005

Serial No.: 09/924,125

Amendments to the Specification:

On page 16, please replace the paragraph extending from lines 25-26 with the following replacement paragraph:

Figures 1A to 1C represent ~~Figure 1 represents~~ nucleotide (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of the human GPR86 ($P2Y_{13}$) receptor according to the invention.

On page 40 of the specification, please replace the second paragraph with the following replacement paragraph:

The NF-κB binding element has the consensus sequence GGGGACTTTCC (SEQ ID NO: 3). A large number of genes have been identified as NF-κB responsive, and their control elements can be linked to a reporter gene to monitor GPCR activity. A small sample of the genes responsive to NF-κB includes those encoding IL-1β (Hiscott et al., 1993, Mol. Cell. Biol. 13: 6231-6240), TNF-α (Shakhov et al., 1990, J. Exp. Med. 171: 35-47), CCR5 (Liu et al., 1998, AIDS Res. Hum. Retroviruses 14: 1509-1519), P-selection (Pan & McEver, 1995, J. Biol. Chem. 270: 23077-23083), Fas ligand (Matsui et al., 1998, J. Immunol. 161: 3469-3473), GM-CSF (Schreck & Baeuerle, 1990, Mol. Cell. Biol. 10: 1281-1286) and IκBα (Haskill et al., 1991, Cell 65: 1281-1289). Each of these references is incorporated herein by reference. Vectors encoding NF-κB-responsive reporters are also known in the art or can be readily made by one of skill in the art using, for example, synthetic NF-κB elements and a minimal promoter, or using the NF-κB-responsive sequences of a gene known to be subject to NF-κB regulation. Further, NF-κB responsive reporter constructs are commercially available from, for example, CLONTECH.

On page 56 of the specification, please replace the last paragraph with the following replacement paragraph:

Specific oligonucleotide primers were synthesized on the basis of the sequence of the GPR86 human receptor: a sense primer 5'-CCGGAATTCACCATGAACACCACAGTGATGC-3' (SEQ ID NO: 4) and an antisense primer 5'-CTTGTCTAGATCAGCCTAAGGTTATGTTGTC-3' (SEQ ID NO: 5). A polymerase chain reaction (PCR) was performed on three different spleen cDNAs using the Platinum Pfx DNA Polymerase. The amplification conditions were as follows: 94°C, 15 s; 50°C, 30 s; 68°C, 2 min for 35 cycles. Amplifications resulted in a fragment of 1 kilobase containing the entire coding sequence of the GPR86 gene. The coding sequence was then subcloned between the EcoRI and XbaI sites of the bicistronic pEFIN5 expression vector and sequenced on both strands for each of the three cDNAs using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, Great Britain).

Please replace the first paragraph on page 58 with the following replacement paragraph:

Reverse transcription-polymerase chain reaction (RT-PCR) experiments were carried out using a panel of polyA+ RNA (Clontech). The GPR86 primers were as follows: GPR86 sense

radioisotope, fluorescent compound, chemiluminescent compound, enzyme, or enzyme co-factor). The antibodies, monoclonal or polyclonal and its hypervariable portion thereof (FAB, FAB", etc.) as well as the hybridoma cell producing the antibodies are a further aspect of the present invention which find a specific industrial application in the field of diagnostics and monitoring of specific diseases, preferably the ones hereafter described.

Inhibitors according to the invention include but are not limited to labeled monoclonal or polyclonal antibodies or hypervariable portions of the antibodies.

As used herein, the term "transgenic animal" refers to any animal, preferably a non-human mammal, bird, fish or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule This molecule may be integrated within a chromosome, or it may be extra-chromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of one of the subject polypeptide, e.g. either agonistic or antagonistic forms. However, transgenic animals in which the recombinant gene is silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs described below. Moreover, "transgenic animal" also includes those recombinant animals in which gene disruption of one or more genes is caused by human intervention, including both recombination and antisense techniques.

Brief Description of Figures

Figure 1 represents nucleotide and deduced amino acid sequence of the human GPR86 ($P2Y_{13}$) receptor according to the invention.

Figure 2 is a dendrogram representing the structural relatedness of the GPR86 ($P2Y_{13}$) receptor with the other P2Y subtypes.

Figures 3A and 3B represent ~~Figure 3 represent~~ tissue distribution of the human GPR86 ($P2Y_{13}$) receptor.

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Figures 4A to 4C represent respectively:

- concentration-action curves of ADP, 2MeSADP and ADPßS on IP_3 accumulation in 1321N1-Gα16 cells expressing the GPR86 ($P2Y_{13}$) human receptor;
- agonistic effects of ADP, ATP and 2MeSATP on IP_3 accumulation in 1321N1 cells expressing the GPR86 ($P2Y_{13}$) human receptor together with $G\alpha_{16}$, and;
- the effect of pertussis toxin on IP_3 accumulation induced by ADP on 1321N1 cells expressing the GPR86 human receptor together with $G\alpha_{16}$.

Figures 5A and 5B represent respectively a concentration-action curve of ADP on cAMP accumulation in CHO-K1 cells expressing the GPR86 ($P2Y_{13}$) human receptor and the effect of pertussis toxin on cAMP accumulation induced by ADP in CHO-K1 cells expressing the GPR86 ($P2Y_{13}$) human receptor according to the invention.

Figures ~~6 shows~~ 6A and 6B show a western blot analysis of phosphorylated Erk1 and Erk2 proteins in CHO-K1 cells expressing the GPR86 ($P2Y_{13}$) human receptor according to the invention.

Figure 7 shows the structure of ADP.

Detailed Description of the Invention

The invention relates to the discovery that ADP is a natural ligand for the orphan G protein coupled receptor GPR86 and methods of using the binding of this ligand to the receptor in a drug screening method. The known ligand and its interaction with the receptor GPR86 also provides for the diagnosis of conditions involving dysregulated receptor activity. The invention also relates to a kit comprising GPR86 ($P2Y_{13}$) and homologous sequences, its corresponding polynucleotide and/or recombinant cells expressing the polynucleotide, to identify agonist, antagonist and inverse agonists compounds of the receptor polypeptide and/or its corresponding polynucleotide. Such kits are useful for the diagnosis, prevention and/or a treatment of various diseases and disorders.

The invention also relates to novel agonist, antagonist and inverse agonists compounds of the receptor polypeptide and its corresponding polynucleotide, identified according to the method of the invention.

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ATTORNEY DOCKET: 9409/2092

METHODS OF IDENTIFYING A LIGAND, AN AGONIST, AND AN

~~THE NATURAL LIGAND FOR ORPHAN~~ G PROTEIN

ANTAGONIST OF G PROTEIN COUPLED RECEPTOR $P2Y_{13}$

~~COUPLED RECEPTOR GPR86 AND METHODS OF USE~~

Field of the Invention

The present invention is related to the natural ligand for the orphan G protein coupled receptor GPR86 and methods of use.

Background of the Invention and State of the Art

Adenine and uridine nucleotides induce pharmacological and physiological responses through several G-protein-coupled receptors (P2Y) and ligand-gated cation channels (P2X) (1, 2). The P2Y family encompasses two selective purinoceptors: the human $P2Y_1$ and $P2Y_{11}$ receptors which are preferentially activated respectively by ADP and ATP (3-5). Nucleotide receptors responsive to both adenine and uracil nucleotides are the $P2Y_2$ receptor, activated equipotently by ATP and UTP (6, 7) as well as the Xenopus $P2Y_8$ (8) and turkey tp2y receptor (9) activated equally by all triphosphate nucleotides. There are also pyrimidinoceptors: the chicken $P2Y_3$ (10) and human $P2Y_6$ (11-13) receptors activated preferentially by UDP, and the human $P2Y_4$ receptor (13-15) activated preferentially by UTP. All these P2Y subtypes are coupled to the phosphoinositide pathway. The $P2Y_{11}$ and tp2y receptors are additionally coupled respectively to stimulation and inhibition of adenylyl cyclase. Other receptors ($P2Y_5$ (16), $P2Y_7$ (17), $P2Y_9$ and $P2Y_{10}$) have been mistakenly included in the P2Y family (18-20). Recently, a $P2Y_{12}$ subtype has been cloned which corresponds to the platelet ADP receptor previously called P_{2T} (21, 22). It is coupled to an inhibition of adenylyl cyclase and is specifically expressed in the platelets and the brain. Its primary structure is not related to the other P2Y receptors but is related to that of the UDP-glucose receptor (23).

More than 300 G protein coupled receptors (GPCRs) have been cloned thus far and it is generally assumed that well over 1000 such receptors exist. Mechanistically, approximately 30-50% of all clinically relevant drugs act by modulating the functions of various GPCRs (34).

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Advantageously, the BLAST algorithm is employed, with parameters set to default values. ~~The BLAST algorithm is described in detail at http://www.ncbi.nih.gov/BLAST/blast_help.html, which is incorporated herein by reference.~~ The search parameters are defined as follows, and can be advantageously set to the defined default parameters.

Advantageously, "substantial identity" when assessed by BLAST equates to sequences which match with an EXPECT value of at least about 7, preferably at least about 9 and most preferably 10 or more. The default threshold for EXPECT in BLAST searching is usually 10.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Karlin and Altschul (Karlin and Altschul 1990, *Proc. Natl. Acad. Sci. USA* 87:2264-68; Karlin and Altschul, 1993, *Proc. Natl. Acad. Sci. USA* 90:5873-7~~; see http://www.ncbi.nih.gov/BLAST/blast_help.html~~) with a few enhancements. The BLAST programs are tailored for sequence similarity searching, for example to identify homologues to a query sequence. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al* (1994) Nature Genetics 6:119-129.

The five BLAST programs available at http://www.ncbi.nlm.nih.gov perform the following tasks: **blastp** - compares an amino acid query sequence against a protein sequence database; **blastn** - compares a nucleotide query sequence against a nucleotide sequence database; **blastx** - compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database; **tblastn** - compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands); **tblastx** - compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

BLAST uses the following search parameters:

HISTOGRAM - Display a histogram of scores for each search; default is yes. (See parameter H in the BLAST Manual).

FILTER - Mask off segments of the query sequence that have low compositional complexity, as determined by the SEG program of Wootton & Federhen (1993) Computers and Chemistry 17:149-163, or segments consisting of short-periodicity internal repeats, as determined by the XNU program of Claverie & States (1993) Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (~~see http://www.ncbi.nlm.nih.gov~~). Filtering can eliminate statistically significant but biologically uninteresting reports from the blast output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

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Low complexity sequence found by a filter program is substituted using the letter "N" in nucleotide sequence (e.g., "NNNNNNNNNNNNN") and the letter "X" in protein sequences (e.g., "XXXXXXXXX").

Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other programs.

It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. Furthermore, in some cases, sequences are masked in their entirety, indicating that the statistical significance of any matches reported against the unfiltered query sequence should be suspect.

NCBI-gi - Causes NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name..

Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm ~~provided at http://www.ncbi.nlm.nih.gov/BLAST~~. In some embodiments of the present invention, no gap penalties are used when determining sequence identity.

Hybridization

The present invention also encompasses nucleotide sequences that are capable of hybridizing to the sequences presented herein, or any fragment or derivative thereof, or to the complement of any of the above.

Another alternative for monitoring GPR86:ADP interactions uses a biosensor assay. ICS biosensors have been described in the art (Australian Membrane Biotechnology Research Institute; ~~http//www.ambri.com.au/;~~ Cornell B, Braach-Maksvytis V, King L, Osman P, Raguse B, Wieczorek L, and Pace R. "A biosensor that uses ion-channel switches" Nature1997, 387, 580). In this technology, the association of GPR86 and its ligand, is coupled to the closing of gramacidin-facilitated ion channels in suspended membrane bilayers and thus to a measurable change in the admittance (similar to impedence) of the biosensor. This approach is linear over six orders of magnitude of admittance change and is ideally suited for large scale, high throughput screening of small molecule combinatorial libraries. A 10% or greater change (increase or decrease) in admittance in a sample containing a candidate modulator, relative to the admittance of a sample lacking the candidate modulator, indicates that the candidate modulator inhibits the interaction of GPR86 and ADP. It is important to note that in assays testing the interaction of GPR86 with ADP, it is possible that a modulator of the interaction need not necessarily interact directly with the domain(s) of the proteins that physically interact with ADP. It is also possible that a modulator will interact at a location removed from the site of interaction and cause, for example, a conformational change in the GPR86 polypeptide. Modulators (inhibitors or agonists) that act in this manner are nonetheless of interest as agents to modulate the activity of GPR86.

3. It should be understood that any of the binding assays described herein can be performed with a non-ADP ligand (for example, agonist, antagonist, etc.) of GPR86, e.g.. a small molecule identified as described herein or ADP analogues including but not limited to any of the ADP analogues presented in US PAT. NO 5,700,786, a natural or synthetic peptide, a polypeptide, an antibody or antigen-binding fragment thereof, a lipid, a carbohydrate, and a small organic molecule.

Any of the binding assays described can be used to determine the presence of an agent in a sample, e.g., a tissue sample, that binds to the GPR86 receptor molecule, or that affects the binding of ADP to the receptor. To do so, GPR86 polypeptide is reacted with ADP or another ligand in the presence or absence of the sample, and ADP or ligand binding is measured as appropriate for the binding assay being used. A decrease of 10% or more in the binding of ADP or other ligand indicates that the sample contains an agent that modulates ADP or ligand binding to the receptor polypeptide.